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Six in one: cryptic species and a new host record for *Olixon* Cameron (Rhopalosomatidae, Hymenoptera) revealed by DNA barcoding

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Abstract

Olixon testaceum is a widely distributed species of brachypterous parasitoid wasp (Vespoidea: Rhopalosomatidae) occurring in Meso- and South America, but little is known of its biology. Here, the first known host of *O.* ?*testaceum* is identified as the cricket *Anaxipha* sp. (Grylloidea: Trigonidiidae) through DNA barcoding of six *Olixon* larvae and their hosts. Barcoding results also indicated substantial genetic diversity within nominal *O. testaceum* specimens. The number of species and statistical significance of these groups were tested using Maximum Likelihood phylogenies, distance-based cluster analyses, and coalescence models. All analyses revealed at least six distinct lineages, which suggests six or more cryptic species within *O.* ?*testaceum*. Combined with what is currently known about *Rhopalosoma* host use, these results indicate that rhopalosomatids may be generalist rather than specialist parasitoids, and further confirm the benefits of open global collaboration and DNA barcoding in advancing taxonomic knowledge.

Keywords

Cricket-assassin wasp, integrative taxonomy

Introduction

In recent decades, new discoveries have greatly increased our knowledge of the diversity, systematics, and behavior of *Olixon* Cameron, 1887–an historically understudied genus of cricket-assassin wasps (Vespoidea: Rhopalosomatidae) (Townes 1977) (Fig. 1). These unusual brachypterous wasps are rarely seen alive but are now being collected in substantial numbers in pitfall and Malaise traps around the world (Mayhew and Dytham 2008; Lohrmann et al. 2012). These specimens have led to new species descriptions (e.g., Lohrmann and Ohl 2007; Krogmann 2009; Lohrmann et al. 2012; Bulbol et al. 2023), new distributional records (e.g., Ramsdell and Taylor 2006; Wood and Maupin 2007), and behavioral notes (Lohrmann et al. 2014), as well as discussions of *Olixon* biogeography and diversification (Krogmann 2009). However, knowledge of their biology remains limited. Here, we report the cricket *Anaxipha* sp. (Grylloidea: Trigonidiidae) as the first confirmed host of *O.* ?*testaceum* and discuss evidence that six cryptic species are included within the nominal species *O. testaceum* in Meso- and South America.

Of the 74 currently described species of extant Rhopalosomatidae (Lohrmann et al. 2020; Bulbol et al. 2021; Bulbol et al. 2023), only three have confirmed hosts. *Rhopalosoma nearcticum* Brues, 1943 are ectoparasitoids of two cricket genera–*Anaxipha* Saussure, 1874 and *Hapithus* Uhler, 1864 (Grylloidea) (Hood 1913; Gurney 1953; Miller et al. 2019), while a single specimen of *O. australiae* (Perkins) has been reared from a cricket identified only to the subfamily Trigonidiinae (Grylloidea: Trigonidiidae) (Perkins 1908). Based upon the examination of museum specimens, Townes (1977), it has been speculated, based on the size of the larvae found attached to hosts, that *O. banksii* (Brues, 1922) may parasitize nemobiine crickets while the scaly cricket, *Cycloptilum trigonipalpum* (Rehn & Hebard, 1912) (Mogoplistidae), may be host for *O. testaceum* Cameron. The observation of itsfemale attacking a nemobiine cricket (Lohrmann et al. 2014) added the first direct evidence for Townes' speculation regarding the host of *O. banksii*.

Despite their brachypterous wings which would seem to limit long-range dispersal, *Olixon testaceum*, as currently understood, is among the most widespread of all rhopalosomatids, occurring throughout Meso- and South America, from Argentina to Arizona (Lohrmann et al. 2012). Specimens are most often collected in Malaise traps and can be found in diverse habitats including rain forests, dry forests, prairies, and cultivated landscapes. The broad distribution of *O. testaceum* coupled with the recent discovery of sympatric cryptic species of *Rhopalosoma nearcticum* in the USA (Miller et al. 2019) made *O. testaceum* an excellent candidate for a species delimitation study using sequence records from the Barcode of Life Database (BOLD; www.boldsystems.org).

An initial search of publicly available rhopalosomatid records revealed 221 sequences from specimens identified as *O.* cf. *testaceum* using the key to species in Lohrmann et al. (2012). Remarkably, four sequences from unidentified rhopalosomatid larvae were also included among these records (Fig. 2). Most of the *O.* cf. *testaceum* and all the larvae originated from the ongoing BioAlfa inventory of the Area de Conservación Guanacaste (ACG) in northwestern Costa Rica in Sector Santa Rosa (Janzen 1986; Janzen and Hallwachs 2016; Janzen et al. 2009). Given the biological significance of new host

Figure 1. A An adult *Olixon* cf. *testaceum* (photo: Paul Bertner) and **B** an *Olixon* cf. *testaceum* 6 larva (BIOUG55891-B08_parasite) attached to its cricket host (BIOUG55891-B08) (photo: CBG Photography Group).

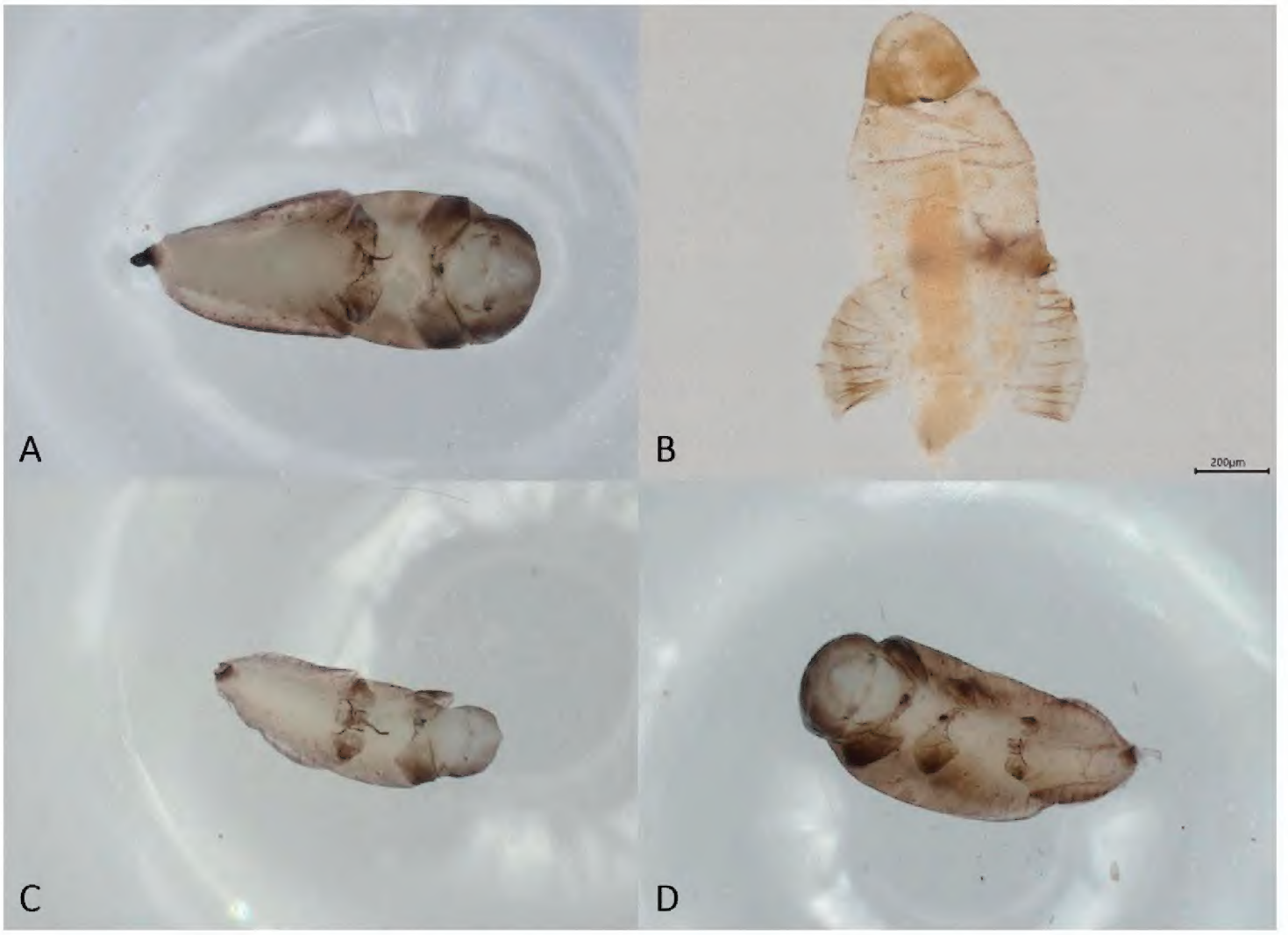

Figure 2. Representative *Olixon* cf. *testaceum* larvae used in this study. BOLD sample IDs **A** BIOUG59151-H08 **B** BIOUG55891-B08_parasite **C** BIOUG63752-H08 **D** BIOUG58943-C02 (photos: CBG Photography Group).

records for Rhopalosomatidae, our objectives were to 1) identify the unknown larvae to species by placing them within a phylogeny of Rhopalosomatidae, 2) identify their host species by searching for associated specimens within BOLD and by generating new barcode sequences as needed, and 3) explore the genetic diversity of *O. testaceum* for evidence of cryptic species.

Methods

Specimens from each sequence cluster in this study were identified to genus using Townes' (1977) key to rhopalosomatid genera and the specimen photos available through BOLD. The subsequent assignment of *Olixon* specimens to the nominal taxon *O. testaceum* was based on the presence of the following combination of characters that distinguishes this species: a more or less uniform testaceous to pale brown coloration with the exception of a dark marking on metasomal segment II, the presence of a malar sulcus, a short temple, and a strong and complete carina between the posterolateral processes of the propodeum (Lohrmann et al. 2012).

The publicly available records (n = 221) were combined with additional private sequences of rhopalosomatids made available by JS, PH, DJ, and WH to ensure maximum coverage, including another two larvae (for a total of six). Specimens were predominantly collected via weekly Malaise trap samples (n = 398) between 2012 and 2020. All but 10 specimens (sourced from GenBank) were sequenced at the Centre for Biodiversity Genomics, and most sequences (n = 309) were generated by a Sequel (Pacific Biosciences) high-throughput sequencer, while 95 were analyzed using Sanger sequencing (https://ccdb.ca/resources/). All sequences, specimen images, and collection data are available in the dataset "DS-RHOP" on BOLD.

An initial Maximum Likelihood (ML) phylogeny of Rhopalosomatidae was created using RAxML (v.8.2.12) (Stamatakis 2014) through the CIPRES Science Gateway (Miller et al. 2010). Alignments were partitioned by codon position and analyzed using the GTRCAT model of nucleotide substitution. Statistical significance was analyzed using 1000 bootstrap (BS) pseudoreplicates. This tree (not shown) included 424 specimens, of which 221 represented *O. testaceum* and six were larvae. All six larvae were located within clades of nominal *O. testaceum*, five in one clade and one in another. In total, the specimens of *O. testaceum* formed six distinct clades, one of which contained 206 of the 221 specimens. As this group contained very little genetic diversity (average genetic distance = 0.0099), subsequent analyses reduced the number of specimens for this clade was reduced from 206 to five representatives.

To narrow the focus to only larvae and potential cryptic species of *O. testaceum*, a new ML phylogeny was created using all six larvae, specimens from each apparent *O. testaceum* clade, all other *Olixon* specimens available (five in total), and four outgroup specimens (two each for *Liosphex* Townes, 1977 and *Rhopalosoma* Cresson, 1865). Sequences were aligned using MAFFT v.7.450 (Katoh et al. 2002; Katoh and Standley 2013) in Geneious Prime® 2020.0.2 (Biomatters, Auckland, NZ). The presence of pseudogenes in the alignment was checked via translation to amino acids. No stop codons were present. The ML phylogeny was reconstructed as described above. Specimen IDs, collection information, and tentative species groups are found in Table 1. Intra- and interspecific genetic distances (K2P) were calculated in MEGA 11 (Tamura et al. 2021).

Table 1. Specimens used in phylogenetic and statistical analyses. Process and Voucher IDs from BOLD: www.boldsystems.org.

Species	Location	Date Collected	Elevation (M)	Stage	Process ID	Sample ID	BIN
O. ?*testaceum* 1	Cortes, HND	7/2/2014	1219	Adult	GMHJK402-15	BIOUG18597-F10	BOLD:ACE2345
O. ?*testaceum* 1	Cortes, HND	7/27/2012	1219	Adult	GMHDO003-13	BIOUG04583-G09	BOLD:ACE2345
O. ?*testaceum* 1	Cortes, HND	6/18/2015	1219	Adult	GMHMQ600-15	BIOUG26862-E11	BOLD:ACE2345
O. ?*testaceum* 1	Cortes, HND	7/24/2014	1196	Adult	GMHKP138-15	BIOUG19409-D02	BOLD:ACE2345
O. ?*testaceum* 1	Cortes, HND	7/16/2015	1219	Adult	GMHMU283-16	BIOUG28324-G04	BOLD:ACE2345
O. ?*testaceum* 2	ACG, CRI	5/25/2020	1366	Adult	CRALC14238-21	BIOUG72979-B05	BOLD:AEO2513
O. ?*testaceum* 3	ACG, CRI	3/13/2014	853	Adult	JICFX017-16	BIOUG29019-H01	BOLD:ACZ7577
O. ?*testaceum* 3	ACG, CRI	1/9/2014	831	Adult	PLEAI182-19	BIOUG48962-D09	BOLD:ACZ7577
O. ?*testaceum* 3	ACG, CRI	5/12/2014	575	Adult	GMAAT178-16	BIOUG27868-D08	BOLD:ACZ7577
O. ?*testaceum* 3	ACG, CRI	8/10/2015	575	Adult	GMADY103-16	BIOUG28200-H03	BOLD:ACZ7577
O. ?*testaceum* 3	ACG, CRI	4/14/2014	575	Adult	GMAAR037-16	BIOUG28246-C12	BOLD:ACZ7577
O. ?*testaceum* 3	ACG, CRI	1/26/2017	828	Adult	PLVAK389-20	BIOUG55894-F08	BOLD:ACZ7577
O. ?*testaceum* 4	ACG, CRI	1/28/2020	15	Adult	CROAC13695-21	BIOUG68837-C03	BOLD:AEM2374
O. ?*testaceum* 4	ACG, CRI	3/3/2022	62	Adult	CROCA33528-21	BIOUG68316-G10	BOLD:AEM2374
O. ?*testaceum* 5	Paramaribo, SUR	10/2/2017	n/a	Larva	GMSPA14567-21	BIOUG70270-H11	BOLD:AEK9228
O. ?*testaceum* 6	ACG, CRI	8/5/2012	300	Adult	GMCRH028-13	BIOUG05414-E09	BOLD:ACG4885
O. ?*testaceum* 6	ACG, CRI	1/18/2018	828	Adult	PLBCJ264-20	BIOUG57554-G01	BOLD:ACG4885
O. ?*testaceum* 6	ACG, CRI	8/30/2018	811	Adult	PLEFA082-21	BIOUG64629-H07	BOLD:ACG4885
O. ?*testaceum* 6	ACG, CRI	5/26/2020	15	Adult	CROAD12739-22	BIOUG80688-H11	BOLD:ACG4885
O. ?*testaceum* 6	ACG, CRI	6/16/2020	15	Adult	CROAD18508-22	BIOUG81047-E07	BOLD:ACG4885
O. ?*testaceum* 6	ACG, CRI	1/19/2017	828	Larva	PLVAJ397-22	BIOUG55891-B08_ parasite	BOLD:ACG4885
O. ?*testaceum* 6	ACG, CRI	8/20/2020	791	Larva	PLDFN085-21	BIOUG63752-H08	BOLD:ACG4885
O. ?*testaceum* 6	ACG, CRI	8/27/2020	811	Larva	PLEFO304-21	BIOUG59841-H04	BOLD:ACG4885
O. ?*testaceum* 6	ACG, CRI	1/18/2018	809	Larva	PLKCJ206-20	BIOUG59151-H08	BOLD:ACG4885
O. ?*testaceum* 6	ACG, CRI	8/30/2018	809	Larva	PLKDP220-20	BIOUG58943-C02	BOLD:ACG4885
O. banksii	IO, USA	22/8/2009	–	Larva	Olixon_Larva_IO	–	
O. banksii	TX, USA	7/6/2011	81	Adult	BBHYA2946-12	BIOUG02644-C10	BOLD:ACA7258
O. banksii	VA, USA	9/28/1993	–	Adult	SICOD002-19	CCDB-34061-A02	BOLD:AEA2163
O. banksii	OK, USA	6/19/2011	–	Adult	BBHYA2958-12	BIOUG02644-D10	BOLD:ACA7139
Olixon sp.	WA, AUS	11/21/2014	–	Adult	GMCWM011-15	BIOUG23860-E06	BOLD:ACZ3980
Liosphex sp.	ACG, CRI	4/28/2014	575	Adult	GMAAS028-16	BIOUG28345-C04	BOLD:ADA1369
Liosphex sp.	ACG, CRI	6/11/2015	1220	Adult	GMCCI021-17	BIOUG36436-H10	BOLD:ADL6377
Rhopalosoma sp.	ACG, CRI	7/12/2018	809	Adult	PLKDI021-20	BIOUG58153-D12	BOLD:ADC7061
Rhopalosoma sp.	ACG, CRI	5/14/2012	300	Adult	GMCGG056-14	BIOUG17755-D09	BOLD:ACG8319

To test for potential cryptic species diversity within *O. testaceum*, both distance-based cluster analyses and phylogenetically informed tests of species delimitation were employed. Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al. 2021) is a distance-based method that tests various species hypotheses using the intra- and inter-genetic distance scores for putative species to calculate a custom ASAP score for each hypothesis (lower scores = more statistical robustness). The online version of ASAP (https://bioinfo.mnhn.fr/abi/public/asap, last accessed Dec. 12, 2022) was used under default settings with genetic distances calculated using the K2P model of molecular evolution.

Additional distance-based cluster analyses were carried out in R (Paradis et al. 2005; R Core Team 2017; Paradis and Schliep 2018). The "dist.DNA" function with model = "TN93" was used to calculate a corrected distance matrix from the imported nucleotide alignment. TN93 was used as it most closely approximates the more complex GTR

model used in the ML analyses which is unavailable using dist.DNA. Hierarchical clustering, which iteratively combines taxa with minimal dissimilarity to create a dendrogram of potentially statistically significant clusters, was performed within the function "parPvclust" using the correlation method and average linkage agglomeration (Suzuki and Shimodaira 2006). The statistical significance of the clusters was confirmed by 1000 bootstrap replicates and the calculation of Approximately Unbiased p-values (AU) and Bootstrap Probability (BP) values as suggested by Suzuki and Shimodaira (2006). The distance matrix was also analyzed using partitioning around medoids, which creates and scores clusters by collapsing the distance of intra-cluster points to a hypothetical centroid. Statistical significance is inferred from average silhouette width of clusters (>0.5 is considered significant) (Kaufman and Rousseeuw 1987). (The "pam" function (Schubert and Rousseeuw 2019) and variable k values ranging from 2 to 8 were used. Results were visualized using "fviz_silhouette" (Kassambara and Mundt 2020).

Phylogenetically informed species delimitation methods were also used. The Multi-rate Poisson Tree Process (mPTP) introduced by Kapli et al. (2017) uses maximum likelihood and is based on a single-locus coalescent-based method. The online version of mPTP (https://mptp.h-its.org/#/tree, last accessed Dec. 12, 2022) was used with default parameters. For a Bayesian analysis, the Bayesian Poisson Tree Process (bPTP) program of Zhang et al. (2013) (https://species.h-its.org/ptp/, last accessed Dec. 12, 2022) was used. The rooted phylogeny was uploaded and analyzed for 250,000 MCMC generations, thinning was set to 150, and the first 25% were discarded as burn-in. Within the program, a simple heuristic search determined the most likely number of species represented on the tree according to the most supported partition. Convergence was verified by visually checking the likelihood plot.

High throughput sequencing (HTS) has the advantage of generating sequences of biota associated with the target specimen. This property enables the identification of potential host-parasitoid interactions, predator-prey relationships, pathogen infections, etc. If a specimen yields more than one sequence contig, the additional contig(s) can be examined for biologically relevant associations. To identify the host for each larva, we compared any additional sequence information generated by the HTS to BOLD and generated a new barcode record when a potential orthopteran host sequence was found.

Results

Thirty-four sequences were used to generate the ML tree (Fig. 3). All sequences are publicly available in BOLD (dataset "DS-RHOP") and identification codes are listed in Table 1. Within *Olixon*, two major lineages were recovered, one including 25 specimens of *O. testaceum* and the other primarily composed of *O. banksii* specimens from the USA. The single specimen of *Olixon* from Australia was recovered as sister to *O. banksii*. Six clades were well-resolved (BS = 100) within *O. testaceum* (informal species IDs are included in Table 1). All rhopalosomatid larvae from Costa Rica were recovered as members of the group "*O. ?testaceum* sp. 6", thus confirming *Anaxipha*

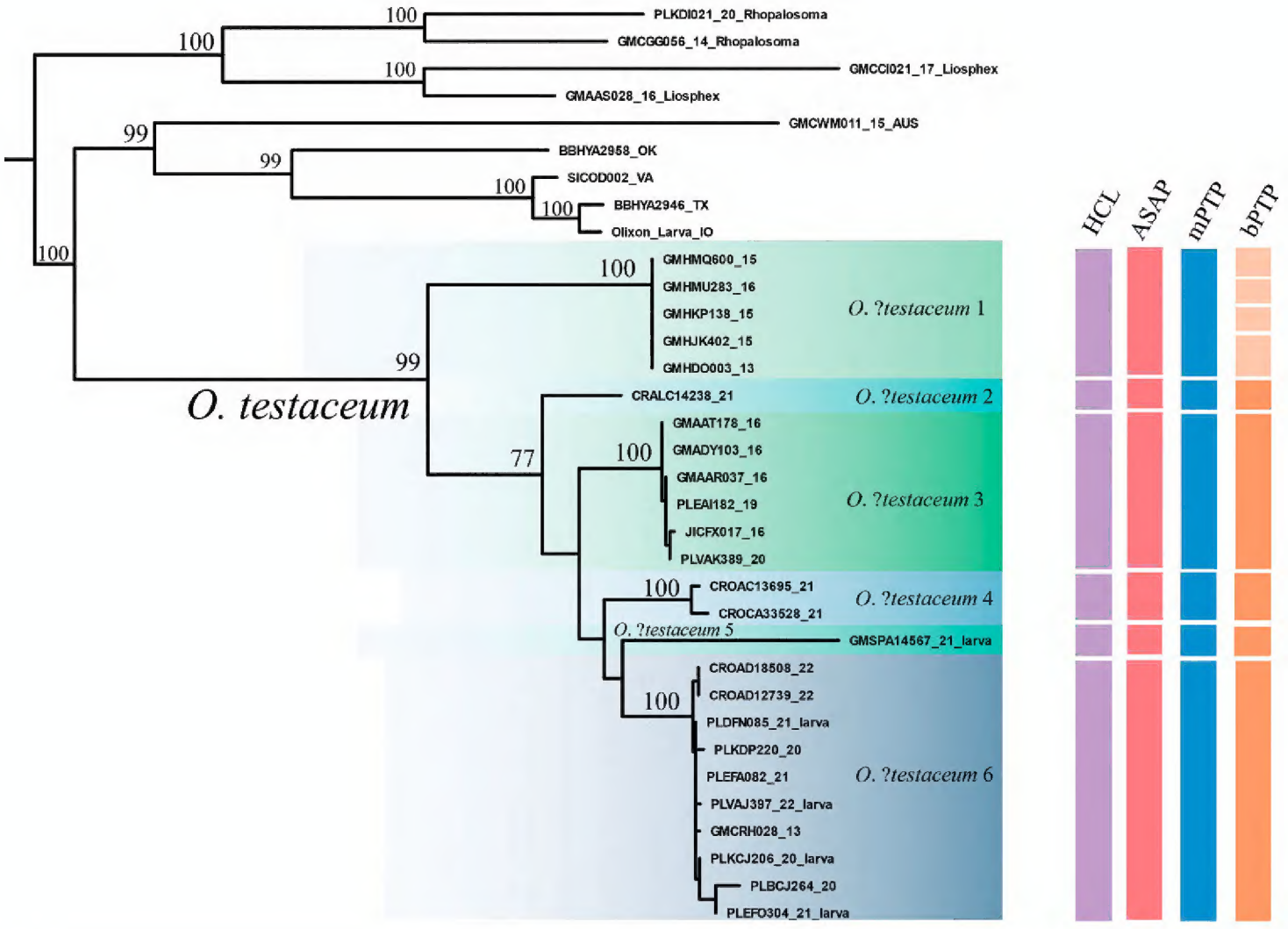

Figure 3. Maximum Likelihood phylogeny of *Olixon testaceum*. Bootstrap support >75 is shown. Putative cryptic species of *O. testaceum* are labeled. Vertical bars indicate statistically significant species groups identified by Hierarchical clustering (HCL), Assemble Species by Automatic Partitioning (ASAP), Multi-rate Poisson Tree Process (mPTP), and Bayesian Poisson Tree Process (bPTP). Light orange bars for bPTP are not significant.

(Trigonidiidae) as a common host for *Olixon*. All novel or newly associated host specimens of *Olixon* are summarized in Table 2.

One larva (BIOU70270-H11) was collected in Suriname. It was found without a host and no associated specimens are confirmed at this time. The other five larvae were collected in ACG, Costa Rica. Two (BIOUG55891-B08_parasite and BIOUG63752-H08) were still attached to their host and barcodes for all four specimens were recovered. Both hosts were identified as *Anaxipha* sp. (Trigonidiidae). Three specimens (BIOUG59841-H04, BIOUG59151-H08, and BIOUG58943-C02) were not attached to a host, likely reflecting their detachment upon exposure to the ethanol in the Malaise trap. Potential orthopteran hosts associated with these samples (i.e., those collected from the same site, date, and trap) were all identified via barcodes as trigonidiid crickets.

All statistical analyses confirmed the presence of at least six distinct lineages within this dataset (Fig. 3), a conclusion reinforced by their differing Barcode Index Numbers on BOLD which often correspond to species (Ratnasingham and Hebert 2013). Hierarchical clustering results and PAM scores (Fig. 4) were statistically significant (AU scores >95, average silhouette width = 0.87). The lowest ASAP-score belonged to the

Table 2. All novel host associations of *Olixon*. Sample IDs from BOLD: www.boldsystems.org.

Species	BIN	Location	Larva Sample ID	Host Sample ID	Host Species	Host BIN	Status
O. ?*testaceum* 5	BOLD:AEK9228	Paramaribo, SUR	BIOUG70270-H11	–	–		–
O. ?*testaceum* 6	BOLD:ACG4885	ACG, CRI	BIOUG55891-B08_parasite	BIOUG55891-B08	*Anaxipha* sp.	BOLD:ACO0556	Known
O. ?*testaceum* 6	BOLD:ACG4885	ACG, CRI	BIOUG63752-H08	BIOUG63752-H08.NTS	*Anaxipha* sp.	BOLD:ACO0556	Known
O. ?*testaceum* 6	BOLD:ACG4885	ACG, CRI	BIOUG59841-H04	BIOUG59841-H05	Trigonidiidae	BOLD:ACG0099	Associated
O. ?*testaceum* 6	BOLD:ACG4885	ACG, CRI	BIOUG59151-H08	BIOUG59151-H08.NTS	*Anaxipha* sp.	BOLD:ACO0556	Associated?
O. ?*testaceum* 6	BOLD:ACG4885	ACG, CRI	BIOUG58943-C02	BIOUG58943-C02.NTS	Trigonidiidae		Associated?

Table 3. Mean pairwise intra- and interspecific genetic distances (K2P) between sampled *Olixon testaceum* specimens. Bold values = intraspecific genetic distance. "n/a" = not applicable due to single taxon.

O. ?*testaceum* 1	**0.000**					
O. ?*testaceum* 2	0.138	**n/a**				
O. ?*testaceum* 3	0.149	0.072	**0.003**			
O. ?*testaceum* 4	0.144	0.071	0.074	**0.010**		
O. ?*testaceum* 5	0.159	0.107	0.113	0.104	**n/a**	
O. ?*testaceum* 6	0.149	0.079	0.063	0.061	0.103	**0.006**

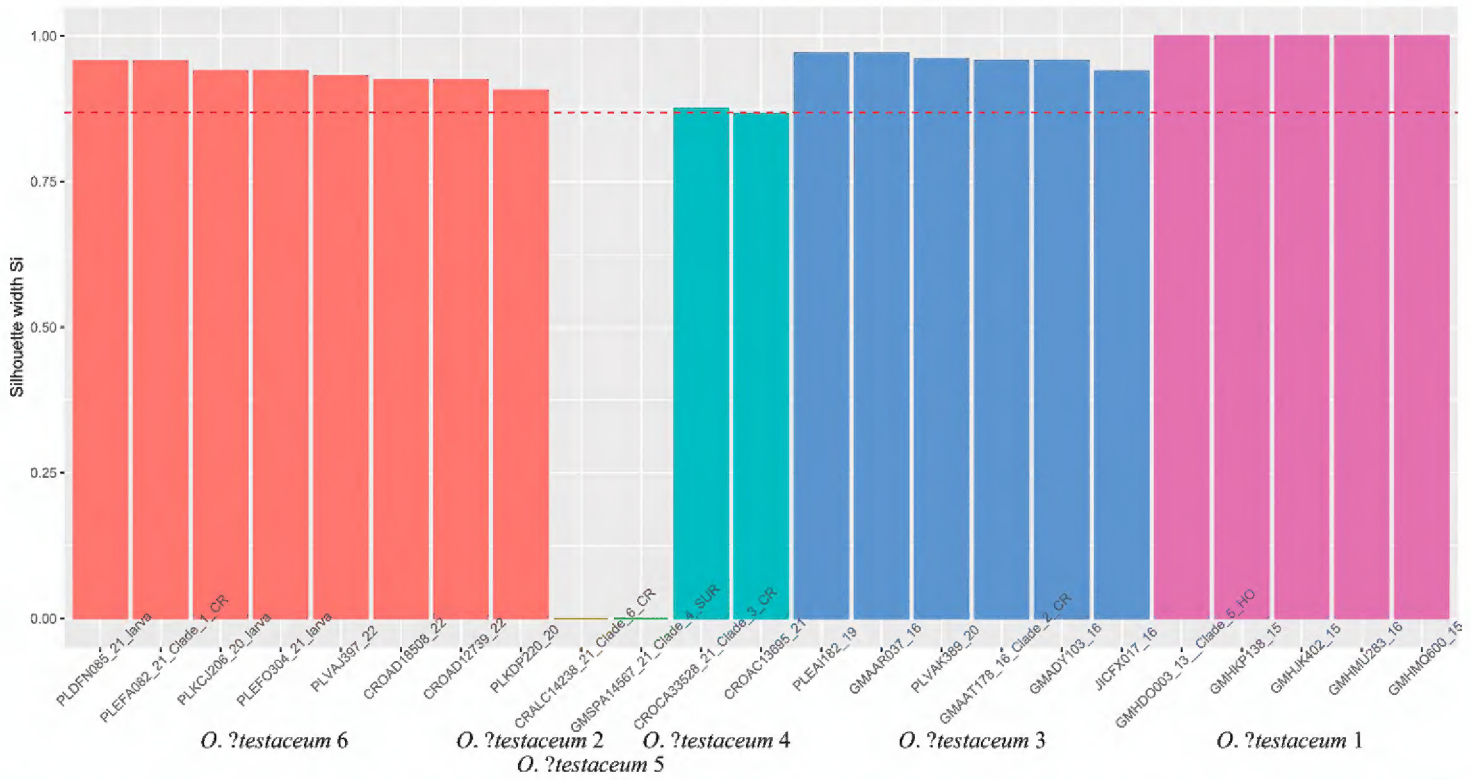

Figure 4. Partitioning Around Medoids analysis of *Olixon* ?*testaceum*. Average silhouette width = 0.87. Statistically significant clusters of *O.* ?*testaceum* are labeled in correlation to ML phylogeny.

partition assuming six hypothetical species (ASAP-score = 1.0; threshold distance = 3.2%). The mPTP suggested six unique lineages, while the bPTP results supported at least six, and possibly up to nine species within *O. testaceum*, although species 7–9 were recovered with very low support. Genetic distances between and within species (Table 3) show very little intraspecific variation per clade (average = .005) and substantial interspecific variation (average = .106).

Discussion

Our analyses indicate several genetic lineages fall within the morphological variation of "*Olixon testaceum*." However, since the holotype has not been barcoded, we are unable to link any particular genetic lineage with the "real" *O. testaceum*. To indicate this uncertainty and acknowledge that future morphological work is needed to formally describe these cryptic species, we refer to the species group collectively as "*O.* ?*testaceum*."

Although *O.* ?*testaceum* is one of the most widespread morphospecies of Rhopalosomatidae, very little is known about its biology (Lohrmann et al. 2012). Here, we have confirmed one new host record for the genus *Olixon* and added several new associated host records for the *O. ?testaceum* species group. At the family level, a new host record for *O. ?testaceum* adds a fourth confirmed host for rhopalosomatid species (Lohrmann et al. 2014; Miller et al. 2019).

Olixon is now known to parasitize species within two subfamilies of crickets: Trigonidiinae (herein, Perkins 1908) and Nemobiinae (*O. banksii*, Lohrmann et al. 2014). Similarly, the only other rhopalosomatid with known hosts, *Rhopalosoma ?nearcticum*, parasitizes both Trigonidiinae and Podoscirtinae crickets (Miller et al. 2019).

The finding of two different subfamilies of crickets as hosts each for *Olixon* and *Rhopalosoma* supports the hypothesis that rhopalosomatids, at least at the genus level, are generalist rather than specialist parasitoids.

Furthermore, the identification of *Anaxipha* as a host of *O.* ?*testaceum* is quite remarkable as *Anaxipha* are also among the known hosts of the distantly related *R.* ?*nearcticum* (Miller et al. 2019). *Olixon* is hypothesized to be the basal branch of Rhopalosomatidae and sister to a clade comprising all recent macropterous forms (Guidotti 1999; Lohrmann et al. 2020) (Fig. 5). The basal position of *Olixon* coupled with the fact that these two rhopalosomatid genera (*Olixon* and *Rhopalosoma*) are not sister taxa but utilize species of the same genus of crickets as hosts may suggest Trigonidiinae as the ancestral host for Rhopalosomatidae. However, other groups of crickets (i.e., Nemobiinae and Podoscirtinae) are also used as hosts by rhopalosomatids and we still know relatively little about host use throughout the family. It is possible the shared trigonidiine host of *Olixon* and *Rhopalosoma* is an example of convergent evolution rather than a plesiomorphy. Unfortunately, hosts for *Paniscomima* Enderlein, 1904 and *Liosphex* are still unknown, but as seen here, large-scale Malaise trap sampling programs are excellent sources for rhopalosomatid adults, larvae, and hosts that may soon fill the gaps in our knowledge of rhopalosomatid biology and evolution of host use.

While a new host record and discovery of cryptic species is significant, there is still much work to be done. Future efforts relating to *O.* ?*testaceum* should investigate morphological or ecological differences that might further distinguish clades from one another. A previous study in the ACG (Hebert et al. 2004), discovered that the target species *Telegonus* (previously *Astraptes*) *fulgerator* (Walch, 1775) contained at least ten cryptic species with defining variation in morphology and host plant preference. Future efforts should investigate whether *O.* ?*testaceum* displays similar variation, especially considering its widespread range. Brief comparison of the six clades revealed by this study showed that *O.* ?*testaceum* sp. 1 from Honduras is easily characterized by its distinct wings which seem, in terms of the grade of their reduction, intermediate between *O. melinsula* Lohmann et al., 2012 and *O.* ?*testaceum* (Fig. 6). Formal species descriptions of these genetic clades should employ an integrative taxonomy approach (e.g. Padial et al. 2010)–adding morphological, behavioral, and host data to the genetic data presented here.

Such integrative taxonomic research may reveal more cryptic species within *O. testaceum* beyond those discovered here. In their revision of the New World *Olixon*, Lohrmann et al. (2012) investigated several hundred specimens assigned to *O. testaceum* originating from the southern United States (Arizona) to northern Argentina. The cryptic diversity of *O. testaceum* reported here is certainly an underestimation of this clade's true diversity since all but one of the specimens analyzed were from Honduras and Costa Rica. The holotype of *Olixon testaceum* was collected in Bugaba, Panama (Cameron 1887), but it is not clear whether its barcode would match any of the clades examined in this study. Furthermore, the slightly different color pattern of *Saphobethylus pallidus* Kieffer, 1911 from Teapa in Mexico, currently treated as a synonym of *O. testaceum* (e.g., Turner and Waterston 1917; Townes 1977; Lohrmann et al. 2012), supports the hypothesis that it too represents a distinct species.

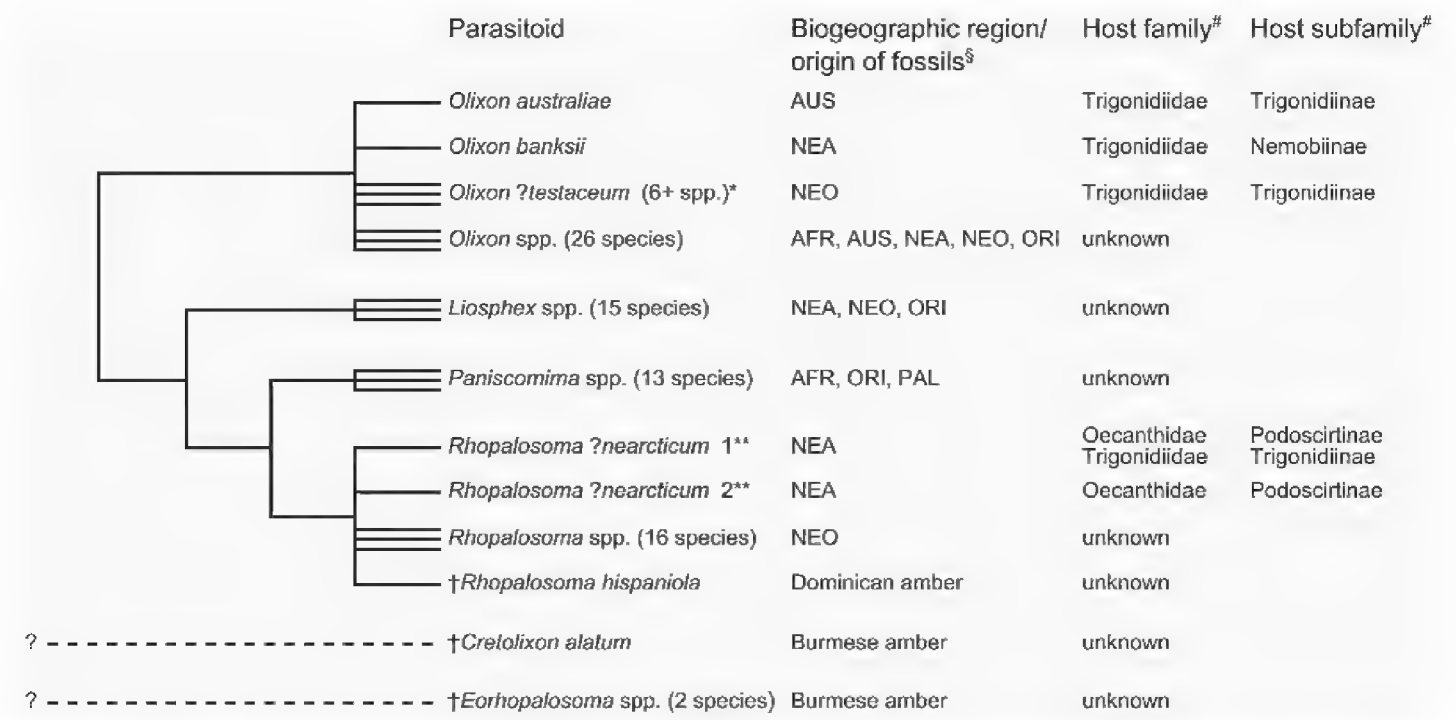

Figure 5. Generalized phylogeny of Rhopalosomatidae showing currently confirmed host associations. The tree topology is based on Brothers (1999), Guidotti (1999), and unpublished molecular data (Blaschke et al., unpublished results). The information on the parasitoid-host-associations are based on Hood (1913), Gurney (1953), and Miller et al. (2019) for *Rhopalosoma cf. nearcticum*, Townes (1977) and Lohrmann et al. (2014) for O. *banksii*, Perkins (1908) for O. *australiae*, and the herein presented data for *O.* cf. *testaceum.* Townes (1977) mentioned *Cycloptilum trigonipalpum* (Mogoplistidae) as the potential host of *O. testaceum*, however, this association is not included here since the association is based only on the size of the wasp larva and the fact that no other *Olixon* species was known from Honduras at that time. Symbols: § Information on the biogeographic regions of the parasitoids are based on Lohrmann et al. (2020; table 1) and Bulbol et al. (2021) for *Liosphex*, Krogmann et al. (2009), Lohrmann et al. (2012), Lohrmann et al. (2020), and Bulbol et al. (2023) for *Olixon*, Lohrmann (2011) and Lohrmann et al. (2020; table 1) for *Paniscomima*, Townes (1977), Miller et al. (2019), and Lohrmann et al. (2019) for *Rhopalosoma*, and Lohrmann et al. (2020) for *Cretolixon* and *Eorhopalosoma*. # The classification of the hosts follows Cigliano et al. (2023). * The herein published data suggests at least six species in the *O. testaceum* species group. **Miller et al. (2019) discovered that the nearctic species *R. nearcticum* actually consists of at least two distinct genetic lineages, i.e., R. ?*nearcticum* 1 and *R.* ?*nearcticum* 2.

Figure 6. *Olixon* spp., female, variations in fore wing morphology **A** *O.* cf. *testaceum* (Costa Rica) **B** *O.* ?*testaceum* 1 (Honduras) **C** *O. melinsula*, paratype (Florida) (photos: Volker Lohrmann).

Rhopalosomatidae appears to be a hotspot for cryptic species diversity. Our ML tree included four specimens of nominal *O. banksii* specimens from Iowa, Texas, Virginia, and Oklahoma. Three specimens from IO, TX, and VA showed low intra-group variation (patristic distance = 0.032), while the average nearest-neighbor distance be-

tween these three and a specimen from OK was significantly higher (patristic distance = 0.234). Another closely related species, *O. melinsula*, is currently known only from Texas to Florida (along the Gulf of Mexico) and southern Paraguay. This distribution pattern may well represent "two sibling species, so similar as to be indistinguishable at the moment" (Lohrmann et al. 2012). Both *O. banksii* and *O. melinsula* are promising groups for future investigations of cryptic species diversity among the cricket-assassin wasps.

The utility of DNA barcoding to identify and reveal cryptic species complexes is well established across a wide range of taxa and biomes (e.g., Hebert et al. 2003; Janzen et al. 2005; Sáez and Lozano 2005; Bickford et al. 2007). Many of the early case studies that applied barcoding to discover cryptic diversity exposed many undescribed species (e.g., Hebert et al. 2004; Smith et al. 2006; Smith et al. 2008). DNA barcoding offers solutions to many of the limitations of a morphologically dependent taxonomic system. Traditional methods for classification require much time and expertise (Stoeckle and Hebert 2008). As barcoding gains wide adoption by both taxonomists and ecologists (Valentini et al. 2009), open collaboration and accessibility to sequences are essential. In order to aid access, BOLD has consolidated this information into a public database with over 15 million specimen records for over 330,000 named species and more than a million putative species.

The taxonomic impediment is a significant and well-known problem in entomology (e.g., Agnarsson and Kunter 2007; Engel et al. 2021) and the results of our study highlight the importance of BOLD's open access policies and friendly collaboration among researchers for the future of insect systematics. In this case, exploring BOLD as part of an undergraduate class revealed unexpected and interesting discoveries in rhopalosomatid host use. New collaborators enthusiastically joined the project, freely sharing specimens, photographs, and their expertise in rhopalosomatid systematics and morphology, DNA barcoding, and species delimitation. We hope future researchers will be similarly generous and collaborative across disciplines and skill levels, allowing for a deeper scientific understanding of the diversity of life and inspiring the next generation of insect taxonomists.

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References

Agnarsson I, Kuntner M (2007) Taxonomy in a changing world: seeking solutions for a science in crisis. Systematic Biology 56(3): 531–539. https://doi.org/10.1080/10635150701424546

Bickford D, Lohman D, Sodhi N, Peter KL, Meier R, Winker K, Ingram K, Das I(2007) Cryptic species as a window on diversity and conservation. Trends in Ecology and Evolution 22(3): 148–155. https://doi.org/10.1016/j.tree.2006.11.004

Brothers DJ (1999) Phylogeny and evolution of wasps, ants and bees (Hymenoptera, Chrysidoidea, Vespoidea and Apoidea). Zoologica Scripta 28(1–2): 233–250. https://doi.org/10.1046/j.1463-6409.1999.00003.x

Bulbol MM, Bartholomay PR, de Oliveira, ML, Somavilla A (2023) A new species of *Olixon* Cameron, 1887 (Hymenoptera: Rhopalosomatidae) and new records for the genus in Brazil. Zootaxa 5277(3): 573–580. https://doi.org/10.11646/zootaxa.5277.3.9

Bulbol MM, Somavilla A, Fernandes DRR, Bartholomay PR (2021) A new species of *Liosphex* Townes, 1977 (Hymenoptera: Rhopalosomatidae) from the Amazon forest and new records for the genus in Brazil. Neotropical Entomology 50(3): 444–452. https://doi.org/10.1007/s13744-021-00862-6

Cigliano MM, Braun H, Eades DC, Otte D (2023) *Orthoptera Species File*. Version 5.0/5.0. [30.05.2023]. http://Orthoptera.SpeciesFile.org

Engel MS, Ceríaco LMP, Daniel GM, Dellapé PM, Löbl I, Marinov M, Reis RE, Young MT, Dubois A, Agarwal I, Lehmann A P, Alvarado M, Alvarez N, Andreone F, Araujo-Vieira K, Ascher JS, Baêta D, Baldo D, Bandeira SA, Barden P, Barrasso DA, Bendifallah L, Bockmann FA, Böhme W, Borkent A, Brandão CRF, Busack SD, Bybee SM, Channing A, Chatzimanolis S, Christenhusz MJM, Crisci JV, D'elía G, Da Costa LM, Davis SR, De Lucena CAS, Deuve T, Elizalde SF, Faivovich J, Farooq H, Ferguson AW, Gippoliti S, Gonçalves FMP, Gonzalez VH, Greenbaum E, Hinojosa-Díaz IA, Ineich I, Jiang J, Kahono S, Kury AB, Lucinda PHF, Lynch JD, Malécot V, Marques MP, Marris JWM, Mckellar RC, Mendes LF, Nihei SS, Nishikawa K, Ohler A, Orrico VGD, Ota H, Paiva J, Parrinha D, Pauwels OSG, Pereyra MO, Pestana LB, Pinheiro PDP, Prendini L, Prokop J, Rasmussen C, Rödel M-O, Rodrigues MT, Rodríguez SM, Salatnaya H, Sampaio Í, Sánchez-García A, Shebl MA, Santos BS, Solórzano-Kraemer MM, Sousa ACA, Stoev P, Teta P, Trape J-F, Dos Santos CVD, Vasudevan K, Vink CJ, Vogel G, Wagner P, Wappler T, Ware JL, Wedmann S, Zacharie CK (2021) The taxonomic impediment: a shortage of taxonomists, not the lack of technical approaches. Zoological Journal of the Linnean Society 193, 381–387. https://doi.org/10.1093/zoolinnean/zlab072

Guidotti AE (1999) Systematics of little known parasitic wasps of the family Rhopalosomatidae (Hymenoptera: Vespoidea). MSc. Thesis, University of Toronto, Toronto.

Gurney AB (1593) Notes on the biology and immature stages of a cricket parasite of the genus *Rhopalosoma*. Proceedings of the United States National Museum 103(3313): 19–34. https://doi.org/10.5479/si.00963801.103-3313.19

Hebert PDN, Penton E, Burns, J (2004) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. PNAS 101(41): 14812–14817. https://doi.org/10.1073/pnas.0406166101

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proceedings: Biological Sciences 270(1512): 313-321. https://doi.org/10.1098/rsbp.2002.2218

Hood JD (1913) Notes on the life history of *Rhopalosoma poeyi* Cresson. Proceedings of the Entomological Society of Washington 15: 145–147.

Janzen DH (1986) Guanacaste National Park: tropical ecological and cultural restoration. In Rehabilitating Damaged Ecosystems 2: 143–192. https://copa.acguanacaste.ac.cr/handle/11606/338

Janzen DH, Hallwachs W (2016) DNA barcoding the Lepidoptera inventory of a large complex tropical conserved wildland, Area de Conservacion Guanacaste, northwestern Costa Rica. Genome 59: 641–660. https://doi.org/10.1139/gen-2016-0005

Janzen DH, Hajibabaei M, Burns J, Hallwachs, W, Remigo E, Hebert PDN (2005) Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. Philosophical Transactions of the Royal Society 360: 1835–1845. https://doi.org/10.1098/rstb.2005.1715

Janzen DH, Hallwachs W, Blandin P, Burns JM, Cadiou J, Chacon I, Dapkey T, Deans AR, Epstein ME, Espinoza B, Franclemont JG, Haber WA, Hajibabaei M, Hall JP, Hebert PD, Gauld ID, Harvey DJ, Hausmann A, Kitching IJ, Lafontaine D, Landry JF, Lemaire C, Miller JY, Miller JS, Miller L, Miller SE, Montero J, Munroe E, Green SR, Ratnasingham S, Rawlins JE, Robbins RK, Rodriguez JJ, Rougerie R, Sharkey MJ, Smith MA, Solis MA, Sullivan JB, Thiaucourt P, Wahl DB, Weller SJ, Whitfield JB, Willmott KR, Wood DM, Woodley NE, Wilson JJ (2009) Integration of DNA barcoding into an ongoing inventory of complex tropical diversity. Molecular Ecology Resources 9: 1–26. https://doi.org/10.1111/j.1755-0998.2009.02628.x

Kapli P, Lutteropp S, Zhang J, Kobert K, Pavlidis P, Stamatakis A, Flouri T (2017) Multi-rate Poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo. Bioinformatics 33(11): 1630–1638. https://doi.org/10.1093/bioinformatics/btx025

Kassambara A, Mundt F (2020) Factoextra: Extract and visualize the results of multivariate data analyses. R Package Version 1.0.7. https://CRAN.R-project.org/package=factoextra

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research 30(14): 3059–66. https://doi.org/10.1093/nar/gkf436

Katoh K, Standley D (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution 30(4): 772–780. https://doi.org/10.1093/molbev/mst010

Kaufman L, Rousseeuw P (1987) Clustering by means of medoids. Statistical Data Analysis Based on the L1-Norm and Related Methods, 405–416. https://wis.kuleuven.be/stat/robust/papers/publications-1987/kaufmanrousseeuw-clusteringbymedoids-l1norm-1987.pdf

Krogmann L, Austin A, Nauman I (2009) Systematic and biogeography of Australian Rhopalosomatid wasps (Hymenoptera: Rhopalosomatidae) with a global synopsis of the enigmatic

genus *Olixon* Cameron. Systematic Entomology 34: 222–251. https://doi.org/10.1111/j.1365-3113.2008.00460.x

Lohrmann V (2011) A revision of the *Paniscomima* of the African subregion with the description of two new species from Malawi and Tanzania (Hymenoptera: Rhopalosomatidae). Zoosystematics and Evolution 87: 371–378. https://doi.org/10.1002/zoos.201100014

Lohrmann V, Fox M, Solis M, Krogmann L (2012) Systematic revision of the New World *Olixon* Cameron with descriptions of *O. melinsula* sp. n. and the hitherto unknown female of *O. bicolor* (Hymenoptera: Rhopalosomatidae). Deutsche Entomologische Zeitschrift 59: 259–275.

Lohrmann V, Falin ZH, Bennet DJ, Engel MS (2014) Recent findings of *Olixon banksii* in the Nearctic with notes on its biology (Hymenoptera: Rhopalosomatidae). Journal of the Kansas Entomological Studies 85: 258–260. https://doi.org/10.2317/JKES130820.1

Lohrmann V, Ohl M (2007) The wasp genus *Olixon* Cameron in Madagascar: first record and description of two new species (Hymenoptera: Rhopalosomatidae). Zootaxa 1465: 39–46. https://doi.org/10.11646/zootaxa.1465.1.4

Lohrmann V, Ohl M, Michalik P, Pitts JP, Jeanneau L, Perrichot V (2019) Notes on rhopalosomatid wasps of Dominican and Mexican amber (Hymenoptera: Rhopalosomatidae) with a description of the first fossil species of *Rhopalosoma* Cresson, 1865. Fossil Record 22: 31–44. https://doi.org/10.5194/fr-22-31-2019

Lohrmann V, Zhang Q, Michalik P, Blaschke J, Müller P, Jeanneau L, Perrichot V (2020) *Cretolixon*-a remarkable new genus of rhopalosomatid wasps (Hymenoptera:Vespoidea: Rhopalosomatidae) from chemically tested, mid-Cretaceous Burmese (Kachin) amber supports the monophyly of Rhopalosomatidae. Fossil Record 23: 215–236. https://doi.org/10.5194/fr-23-215-2020

Mayhew P, Dytham C (2008) The effectiveness and optimal use of Malaise traps for monitoring parasitoid wasps. Insect Conservation and Diversity 1: 22–31. https://doi.org/10.1111/j.1752-4598.2007.00003.x

Miller MA, Pfeiffer W, Schwartz T (2010) "Creating the CIPRES Science Gateway for inference of large phylogenetic trees" in Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA, 1–8. https://doi.org/10.1109/GCE.2010.5676129

Miller LA, Benefield TD, Lounsbury SA, Lohrmann V, Blaschke JD (2019) DNA barcoding of rhopalosomatid larvae reveals a new host record and genetic evidence of a second species of *Rhopalosoma* Cresson (Hymenoptera: Rhopalosomatidae) in America north of Mexico. Journal of Hymenopteran Research 74: 35–46. https://doi.org/10.3897/jhr.74.38276

Padial, JM, Miralles A, De la Riva I, Vences M (2010) The integrative future of taxonomy. Frontiers in Zoology 7(1): 1–14. https://doi.org/10.1186/1742-9994-7-16

Paradis E, Schliep K (2018) Ape 5.0: An environment for modern phylogenetics and evolutionary analyses in R. Bioinformatics 35(3): 526–528. https://doi.org/10.1093/bioinformatics/bty633

Paradis E, Claude J, Strimmer K (2005) APE: Analyses of phylogenetics and evolution in R language. Bioinformatics 20(2): 289–290. https://doi.org/10.1093/bioinformatics/btg412

Perkins RCL (1908) Some remarkable Australian Hymenoptera. Proceedings of the Hawaiian Entomological Society 2: 27–35. http://biostor.org/reference/58184

Puillandre N, Brouillet S, Achaz G (2021) ASAP: assemble species by automatic partitioning. Molecular Ecology Resources 21(2): 609–620. https://doi.org/10.1111/1755-0998.13281

Ramsdell KMM, Taylor SJ (2006) A new state record for *Olixon Banskii* Brues (Hymenoptera: Rhopalosomatidae) in Indiana, USA. Entomological News 17(3): 351–352. https://doi.org/10.3157/0013-872X(2006)117[351:ANSRFO]2.0.CO;2

Ratnasingham S, Hebert PDN (2013) A DNA-based registry for all animal species: the Barcode Index Number (BIN) system. PloS ONE 8(7): e66213. https://doi.org/10.1371/journal.pone.0066213

R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Sáez A, Lozano E (2005) Body doubles. Nature 433: 111. https://doi.org/10.1038/433111a

Schubert E, Rousseeuw P (2019) Faster k-Medoids Clustering: Improving the PAM, CLARA, and CLARANS Algorithms. SISAP 2020 171–187. https://doi.org/10.1007/978-3-030-32047-8_16

Smith MA, Woodley NE, Janzen DH, Hebert PDN (2006) DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). PNAS 103(10): 3657–3662. https://doi.org/10.1073/pnas.0511318103

Smith MA, Rodriguez JJ, Whitfield JB, Deans AR, Hanzen DH, Hallwachs W, Hebert PDN (2008) Extreme diversity of tropical parasitoid wasps exposed by interactive integration of natural history, DNA barcoding, morphology, and collections. PNAS 105(34): 12359–12364. https://doi.org/10.1073/pnas.080531910

Stamatakis A (2014) RaxML version 8:l a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30(9): 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Stoeckle M, Hebert PDN (2008) Barcode of life. Scientific American 299(4): 82–88. https://doi.org/10.1038/scientificamerican1008-82

Suzuki R, Shimodaira H (2006) Pvclust: An R Package for assessing the uncertainty in hierarchical clustering. Bioinformatics 22(12): 1540–1542. https://doi.org/10.1093/bioinformatics/btl117

Tamura K, Stecher G, Kumar S (2021) MEGA11: molecular evolutionary genetics analysis version 11. Molecular Biology and Evolution 38: 3022–3027. https://doi.org/10.1093/molbev/msab120

Townes H (1977) A revision of the Rhopalosomatidae (Hymenoptera). Contributions of the American Entomological Institute 58: 173–321. https://agris.fao.org/agris-search/search.do?recordID=US19810690678

Turner RE, Waterston J (1917) Notes on the hymenopterous families Bethylidae and Rhopalosomidae. Annals and Magazine of Natural History 8(20): 101–108. https://doi.org/10.1080/00222931709486976

Valentini A, Pompanon F, Taberlet P (2009) DNA barcoding for ecologists. Trends in Ecology and Evolution 24(2): 110–117. https://doi.org/10.1016/j.tree.2008.09.011

Wood DL, Maupin MA (2007) A new state record for *Olixon banksii* (Hymenoptera: Rhopalosomatidae) in Missouri. The Great Lakes Entomologist 40(1–2): 101–102. https://doi.org/10.22543/0090-0222.2181

Zhang J, Kapli P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29 (22): 2869–2876. https://doi.org/10.1093/bioinformatics/btt499